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(54) Title: DRUG PREPARATIONS OF REDUCED TOXICITY (57) Abstract Preparations of drugs in admixture with certain ligands which, when administered to animals or humans, are less toxic than conventional drug preparations. Although the toxicity of the drug-ligand preparations described is greatly reduced, the drug retains pharmacological activity.		

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DRUG PREPARATIONS OF REDUCED TOXICITY

1. FIELD OF THE INVENTION

30 This invention relates to reducing or "buffering" the toxicity of drugs whose side effects are mediated through binding to endogenous cellular toxicity receptors, generally lipids. In particular, the drug is administered

in combination with certain ligands such that the toxic effects of the drug are greatly reduced; however, the drug retains pharmacological activity.



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2. BACKGROUND OF THE INVENTION

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Several theories have been advanced to explain the mechanism by which a variety of toxic substances exert their desirable therapeutic and detrimental toxic effects in animals, including humans. Growing evidence suggests that these substances exert their effects by disturbing the cellular membranes (Schacht et al., 1983, "Aminoglycoside-cell Receptor Interactions: Implications for Toxicity and In Vitro Models," Thirteenth International Congress of Chemotherapy). More particularly, it is believed that the drugs bind to certain specific receptors which mediate the molecular activities responsible for their therapeutic and toxic effects in animals.

Theories of pharmacological action have been developed for the aminoglycoside antibiotics and for several other drugs, including the polyene and polyene macrolide antibiotics, a class of antifungal agents, as well as for adriamycin and cisplatin, known antineoplastic agents. These drugs, among others, exert their desirable therapeutic effects by binding to specific bacterial, fungal or tumor cellular receptors. Their undesirable toxic effects, on the other hand, are thought to be due to binding to specific toxicity receptors of non-drug target cells of the treated animal. Lipids have been suggested as toxicity receptors for these and other drugs.

Since theories have been more developed for the above-listed drugs, these will be discussed in greater



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detail below. It is to be understood, however, that drugs other than these may also exert their toxic effects through similar lipid-mediated mechanisms, and that the present invention encompasses all of such drugs.

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2.1. THE AMINOGLYCOSIDE ANTIBIOTICS

The aminoglycoside antibiotics (e.g., streptomycin, gentamycin, kanamycin, tobramycin, etc.) are used almost exclusively to treat infections caused by bacteria. Their mode of bactericidal action involves inhibition of protein synthesis in susceptible microorganisms. Some susceptible microorganisms include Escherichia spp., Haemophilus spp., Listeria spp., Pseudomonas spp., Nocardia spp., Yersinia spp., Klebsiella spp., Enterobacter spp., Salmonella spp., Staphylococcus spp., Streptococcus spp., Mycobacteria spp., Shigella spp., and Serratia spp., to name but a few.

The antibiotics in the aminoglycoside group all contain amino sugars in glycosidic linkages. They are polycations and their polarity is primarily responsible for the pharmacokinetic properties shared by the members of the group. For instance, these drugs are not adequately absorbed after oral administration, they do not easily penetrate the cerebrospinal fluid, and they are rapidly excreted by the kidney.

Serious toxicity is a major limitation to the usefulness of aminoglycosides. Three types of toxicity are often encountered with the use of aminoglycosides: (1) ototoxicity, which can involve both auditory and vestibular functions of the eighth cranial nerve; (2) nephrotoxicity, which is manifest as acute tubular damage; and (3) acute toxicity, which can follow

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intrapleural and intraperitoneal administration and is manifest as a neuromuscular blockade culminating in respiratory distress.

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Ototoxicity involves labyrinthine dysfunction. Nearly 75% of patients given 2g of streptomycin daily for 60 to 120 days manifest some vestibular disturbance; reduction of the dose to 1g daily decreases the incidence to 25%. The effects occur in stages: (1) the acute stage (1 to 2 weeks) is characterized by nausea, vomiting and equilibratory difficulty including vertigo; (2) the acute state ends suddenly and is followed by chronic labyrinthitis characterized by ataxia; and (3) the chronic stage may persist for two months and is followed by a compensatory stage in which symptoms are latent and appear only when the eyes are closed. Full recovery of coordination may require 12 to 18 months, and some patients have permanent residual damage. There is no specific treatment for the vestibular deficiency.

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In order to prevent or reduce the incidence and severity of the toxic effect on vestibular function, the aminoglycoside dose, the duration of therapy, and careful observation of the effects on the patient must be considered.

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The toxic effect of aminoglycosides such as streptomycin and gentamycin is greater upon the vestibular than auditory component of the eighth cranial nerve, however, a decrease in hearing occurs in an appreciable number of patients (4-15% of individuals receiving the drug for more than 1 week) and, in rare cases, complete deafness can occur. Although the location of lesions responsible for vestibular and auditory dysfunction is disputed, it is believed that aminoglycosides destroy the

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receptors for aminoglycoside (hereinafter referred to as putative aminoglycoside toxicity receptors) and render tissues susceptible to these drugs (Lodhi et al., 1980, Biochem. Pharmacol. 29:597-601).

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See "The Pharmacological Basis of Therapeutics," 6th edition, Goodman and Gilman, eds., 1980, Ch. 51, pp. 1162-1199, for a review of the aminoglycosides.

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2.2. THE POLYENE AND POLYENE MACROLIDE ANTIBIOTICS

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Polyene and macrolide antibiotics are a group of substances obtained from species of actinomycetes. For a review of the pharmacology of various polyene and polyene macrolide antibiotics, see "The Pharmacological Basis of Therapeutics", 6th edition, Goodman and Gilman, eds., 1980, pp. 1233-1236 and Medoff et al., 1983, Ann. Rev. Pharmacol. Toxicol. 23:303-330.

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A large number and variety of untoward toxic effects may be associated with the use of these drugs. In particular, the toxic effects of amphotericin B include anaphylaxis, thrombopenia, flushing, generalized pain, convulsions, chills, fever, phlebitis, headache, anemia, anorexia, and decreased renal function. The toxic effects of the polyene and polyene macrolide antibiotics appear to result from binding to the toxicity receptor, cholesterol, a sterol present in animal cells but not fungal cells.

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The polyene and polyene macrolide antibiotics appear to exert their therapeutic effects against fungi by binding to ergosterol, a sterol present in fungal cells but not in human cells. For example, amphotericin B binds in vitro to ergosterol with approximately ten times greater affinity than to cholesterol, the putative



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blockade has also been observed in man following intravenous, intramuscular, and oral administration of these agents. The neuromuscular block may occur by inhibition of acetylcholine release from the preganglionic terminal (through competition with calcium ions) and perhaps to a lesser extent by stabilization of the post junctional membrane. The blockade is antagonized by calcium salts, but only inconsistently by anticholinesterase agents.

These neuromuscular reactions are shared to varying degrees by several other aminoglycoside antibiotics, particularly neomycin and kanamycin, and to a lesser extent by gentamycin, viomycin, paromomycin and tobramycin. Interestingly, the order of increasing ability of the aminoglycosides to affect acute toxic reactions seems to correlate with their ability to affect nephrotoxicity. That the underlying mechanism of these toxic reactions may be related is suggested by work associating the stimulation or depression of bioelectric events in nerve membranes with phosphoinositide metabolism (Hokin, 1969, Structure and Function of Nervous Tissue, Bourni, ed., Academic Press, New York, Vol. 3, pp. 161-184; Schact and Agranoff, 1972, J. Neurochem. 19:1417-1421; Margolis and Heller, 1966, J. Pharmacol. Expt. Ther. 74:307-312) and similar work associating phosphoinositide metabolism with aminoglycoside-induced ototoxicity and nephrotoxicity (Orsulakova et al., 1976, J. Neurochem. 26:285-290; Schibeci and Schacht, 1977, Biochem. Pharmacol. 26:1769-1774; Alexander et al., 1979, J. Antibiotics 32:504-510). Aminoglycoside antibiotics have been shown to bind to polyphosphoinositides in inner ear tissue and kidney in vivo. It has been postulated that phosphatidylinositol bisphosphates (phosphatidylinositol diphosphate) serve as in vivo



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ventral cochlear nuclei in the brain stem with extension of pathological changes to the terminals of the nerve fibers in the cochlea.

5 Nephrotoxicity caused by aminoglycosides is essentially a form of acute tubular necrosis and is initially manifested by the inability to concentrate urine. The very high concentrations of aminoglycoside antibiotics which accumulate in the renal cortex and urine
10 correlate with the potential of these drugs to cause nephrotoxicity. For this reason, neomycin, the most nephrotoxic aminoglycoside, is not generally administered systemically in humans. Gentamycin seems to be the most
15 nephrotoxic of the commonly used drugs.

Typically, after 5 to 7 days of aminoglycoside therapy, kidney damage, manifested by acute tubular necrosis and inability to concentrate urine, occurs and progresses as administration of the drug continues. At
20 this stage, the urine contains protein and tubular cell casts. A reduction in glomerular filtration rate follows and is associated with elevation in the concentrations of the aminoglycoside, creatinine, and urea in plasma. Histopathology includes acute tubular damage with
25 secondary interstitial damage. These changes are usually reversible and regeneration of renal cells occurs if the drug is discontinued.

Another serious effect of various aminoglycosides
30 is the potentially fatal neuromuscular reaction which may develop when a drug such as streptomycin is instilled into the peritoneal cavity postoperatively (a practice in some surgical clinics). Acute muscular paralysis and difficulty in respiration may occur due to blockade of the
35 neuromuscular junction by aminoglycosides. Neuromuscular



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toxicity receptor. Bittman et al., 1982, Biochim. Biophys. Acta 685:219.

5 Encapsulating amphotericin B in liposomes has
been suggested to attenuate the untoward effects of the
drug. Taylor et al. (1982, Ann. Rev. Respir. Dis.
125:610-611) reported that liposome encapsulation
significantly alters the drug's toxic effects. Their data
10 indicates that encapsulation probably changes the tissue
distribution of the drug. They observed reduced acute
toxicity and a maximal tolerable dose nine times greater
than the maximal tolerable dose for free amphotericin B.
Liposome encapsulation of amphotericin B also prolongs the
survival of mice infected with Histoplasma capsulatum.
15 New et al., (1981, J. Antimicrob. Chemother. 8:371-381)
have used amphotericin B with cholesterol for the purpose
of treating leishmaniasis. Increased effectiveness of the
drug was observed.

20 Sodium deoxycholate is used in a commercial
preparation, Fungizone, to effect a colloidal dispersion
of amphotericin B, buffers and diluents. Fungizone is
available packaged in vials as a sterile, lyophilized
powder to which water is added. This mixture is shaken
25 and added to a 5% dextrose solution. The resulting
solution can then be administered as an injection.

2.3. ADRIAMYCIN

30 Adriamycin, an antineoplastic drug, is a
glycosidic anthracycline antibiotic that is a fermentation
product of the fungus Streptomyces peucetius var.
caesius. Adriamycin has a tetracycline ring structure
with an unusual sugar, daunosamine, attached by a
35 glycosidic linkage.



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Adriamycin is effective in the treatment of acute leukemias and malignant lymphomas and in a number of solid tumors. The drug is particularly beneficial in a wide range of sarcomas, including osteogenic, Ewing's, and soft-tissue sarcomas. It is one of the most active single agents for the treatment of metastatic adenocarcinoma of the breast, carcinoma of the bladder, bronchogenic carcinoma and neuroblastoma.

Myelosuppression is a major dose-limiting complication with adriamycin. Stomatitis, gastrointestinal disturbances, and alopecia are common but reversible. Cardiomyopathy is a unique characteristic of the anthracycline antibiotics. Tachycardia, arrhythmias, and ST-T wave changes in the ECG may be early manifestations of cardiac toxicity. Severe and rapidly progressive congestive heart failure may follow. Nonspecific alterations, including a decrease in the number of myocardial fibrils, mitochondrial changes, and cellular degeneration, are visible by electron microscopy. In view of this cardiotoxicity, therapeutic utility of adriamycin is limited.

Because adriamycin is primarily metabolized and excreted by the liver, it is important to reduce the dosage in patients with impaired hepatic function.

The antitumor effects of adriamycin appear to result from intercalation of the molecule between adjacent base pairs of DNA, thus interfering with the proliferation of rapidly dividing tumor cells. Toxic effects appear to be mediated by binding of the drug to cardiolipin, the putative toxicity receptor. Goormaghtigh et al., have shown that adriamycin adsorbs in vitro to cardiolipin and, to lesser degrees, to phosphatidylserine and phosphatidic



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acid (1980, Biochem. Pharmacol. 29:3003-3010). There appears to be a relatively strong electrostatic interaction between protonated amine groups of the sugar residues of adriamycin and ionized phosphate residues of the cardiolipin, concomitant with interaction between adjacent anthraquinone chromophores. The association constant for cardiolipin/adriamycin is about $1.6 \times 10^6 \text{ mol}^{-1}$, while the association constant for DNA/adriamycin is about $2.4 \times 10^6 \text{ mol}^{-1}$. Thus, adriamycin has a higher affinity for DNA than for cardiolipin.

Fluorescent studies have revealed that adriamycin binds to the head group of cardiolipin. Goormaghtigh et al., 1980, Biochim. Biophys. Acta 597:1-14.

Goormaghtigh et al., also observed a good correlation between binding of adriamycin derivatives to cardiolipin and mitochondrial toxicity (1980, Biochem. Pharmacol. 29:3003-3010). Thus, it has been proposed that cardiolipin is the toxicity receptor for adriamycin in animals (Goormaghtigh et al., 1982, Biochem. Biophys. Res. Comm. 104:314-320).

It has been proposed to encapsulate adriamycin in liposomes (Forssen and Tokes, 1983, Cancer Res. 43:546-550). Liposome encapsulation changes the tissue distribution of adriamycin; the free drug appears predominantly in the liver and spleen. There is an apparent reduction in chronic cardiotoxicity and an increase in antileukemic activity.

2.4. CISPLATIN

Cisplatin is an inorganic, water soluble, platinum containing coordination complex (planar),



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containing ammonium and chloride residues in the cis configuration. Despite pronounced nephrotoxicity and ototoxicity, cisplatin is very useful in combination chemotherapy of metastatic, testicular and ovarian carcinoma. Encouraging effects have also been reported during treatment of tumors of the bladder and of the head and neck.

For a review of the pharmacology of cisplatin, see "The Pharmacological Basis of Therapeutics", 6th edition, Goodman and Gilman, eds., 1980, pp. 1298-1299.

Studies on crystal structure analysis of cisplatin have demonstrated a strong binding of these compounds to guanosine and related inosine derivatives (e.g., inosine 5'-monophosphate) (Louie and Bau, 1977, J. Amer. Chem. Soc. 99:3874-3876; Goodgame et al., 1975, Biochim. Biophys. Acta 378:153; Bau et al., 1977, J. Clin. Hematol. Oncol., Wadley Medical Bulletin 7:51). Thus, the mechanism of action leading to the nephrotoxicity and ototoxicity observed upon administration of cisplatin may be similar to that for the aminoglycoside antibiotics (see Section 2.1.) and ligands useful for buffering aminoglycosides may be used for buffering cisplatin.

2.5. CONCURRENT THERAPY

Combinations of antimicrobial agents have been widely described for the treatment of infections. In fact, combination therapy using a number of different drugs is recommended for the treatment of many cancers.

Concurrent therapy, however, with certain pharmaceutical agents may be complicated because agents which exert a synergistic effect in vitro cannot be



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formulated in a single mixture to use in vitro. Mixtures of gentamycin and nafcillin, a penicillin, at therapeutically effective concentrations result in the formation of complexes that precipitate out of solution and, therefore, cannot be administered in vivo together. In fact, certain drug combinations are not recommended for use in vivo due to drug incompatibility (i.e., either inactivation of the drug or formation of a precipitate). For example, it is recommended that the following antibiotics not be mixed with any other drug: gentamycin, kanamycin, lincomycin, cephalothin, and ampicillin (Davis and Abbitt, 1977, JAVMA 170(2):204-207). Moreover, certain agents cannot be solubilized in the same media due to chemical restraints (e.g., a lipid soluble compound and a water soluble compound).

3. SUMMARY OF THE INVENTION

The present invention relates to preparations of certain drugs in admixture with various ligands that reduce the toxicity of the drugs in vivo. When these drugs are administered in combination with the ligands described herein, toxicity of the drug is greatly reduced while the drug retains pharmacological activity. Drugs whose toxic effects may be reduced or "buffered" by the method of the invention include those in which toxic side effects are mediated through binding to endogenous cellular substrates, which are generally lipids. The term "ligand" as used herein includes compounds which reduce the toxicity of the drugs in the preparation; the term is not meant to indicate the type of interaction or reaction between such compounds and the drug.



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5 A particularly useful advantage of the
drug-ligand preparations described herein is that high
doses of drug may be administered to an animal or human
with lessened deleterious toxic effects than observed with
conventional drug preparations. Thus, maintenance doses
may be given less often to achieve acceptable serum
levels. Since higher doses of drug may be administered
without the usual toxic effects, the preparations of the
invention allow for a greater margin of error in selection
10 of initial doses of drug.

The preparations of the invention can be viewed
as an entirely new class of therapeutic compositions
possessing all of the advantages of the conventional drug
15 preparations with reduction of the major clinically
relevant disadvantages.

4. DETAILED DESCRIPTION OF THE INVENTION

20 The present invention involves drug preparations
of reduced toxicity. A drug-ligand preparation is made
such that, when administered to an animal or human, the
toxicity of the drug in the preparation is reduced or
eliminated, yet the drug retains pharmacological activity.

25 The ligand to be used in the preparations of the
invention depends on the particular drug used and the
relevant toxicity receptor. Generally, the drugs which
may be used in practicing the present invention are those
30 whose in vivo toxicity to mammalian cells is mediated
through binding to lipid toxicity receptors. Such drugs
include monoaminated or polyaminated drugs,
antimicrobials, antivirals, antifungals, antibacterials,
aminoglycosides, polyene and polyene macrolide
35 antibiotics, lincosamides, and polymixins, to name a few.



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According to the invention, appropriate ligands (lipids or lipid head groups) to which the drug binds are admixed with the drug prior to administration. It is believed the ligand binds to the toxicity receptor to prevent interaction between the drug and the receptor. This combination of drug with ligand prevents or reduces the binding of the drug to its endogenous toxicity receptor, thus reducing or eliminating the toxicity of the drug. The drug retains its pharmacological effectiveness.

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4.1. AMINOGLYCOSIDE-LIGAND PREPARATIONS

As discussed in Section 2.1., it is believed that phosphatidylinositol biphosphates serve as in vivo toxicity receptors for the aminoglycoside antibiotics. According to the invention, aminoglycosides are mixed with ligands which prevent or reduce the binding of aminoglycosides to the endogenous toxicity receptors. Ligands useful in the aminoglycoside preparations of the invention include, but are not limited to, certain phosphate esters, phosphate anhydrides and sulfatides.

The ligands which may be used in the present invention are not equally effective in reducing aminoglycoside toxicity (see Section 5.1., infra). The ability of these ligands to reduce toxicity of aminoglycoside antibiotics appears to correlate with two phenomena. The first phenomenon is the degree to which various ligands appear to associate with the aminoglycosides by virtue of the strength of dipole-dipole interaction and subsequent hydrogen bonding. Ligands exhibiting extensive resonance structures, whereby electron delocalization is easily visualized, seem to provide the most protection against aminoglycoside toxicity. Ligands exhibiting lower potential for hydrogen

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bonding provide only intermediate protection. The configuration of negative charges on phosphatidylinositol biphosphate (the putative aminoglycoside toxicity receptor) allow for a specific, three point binding to positively charged aminoglycosides. Although a strong correlation exists between the charge of aminoglycosides and their relative toxicity (Schact, 1983, Proceedings of the Thirteenth International Congress of Chemotherapy), some aminoglycosides do not fit this pattern of charge versus toxicity and, thus may be stabilized in their binding to phosphatidylinositol biphosphate by hydrogen bonding. Thus, the ligands that most effectively reduce the toxicity seem to have the same binding characteristics of phosphatidylinositol biphosphate.

The second phenomenon concerning the efficacy of various ligands to reduce aminoglycoside toxicity concerns a correlation of the ability of these ligands to bind C-reactive protein. C-reactive protein is an acute phase protein appearing in the serum of man during various pathologic conditions. It is precipitated from such serum by pneumococcal C-polysaccharide in the presence of calcium ions. Phosphorylcholine and lysophosphatidylcholine bind strongly to C-reactive protein, while phosphorylethanolamine and glycerolphosphorylcholine bind less strongly (Volanakis and Kaplan, 1970, Proc. Soc. Exp. Biol. Med. 136:612-614). Coincidentally, phosphorylcholine and lysophosphatidylcholine are very effective in reducing aminoglycoside toxicity whereas phosphorylethanolamine and glycerolphosphorylcholine are less effective. The correlation of the ability of these ligands to bind C-reactive protein and the ability to reduce aminoglycoside toxicity while retaining antimicrobial activity remains unexplained. This correlation is useful



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as a predictive tool to select candidates for ligands useful in the preparations of the invention, but it is to be understood that this does not necessarily indicate the mechanism of action by which the preparations of the invention exert the observed effects.

The ligands which are used in the present invention to attenuate aminoglycoside toxicity include, but are not limited to: lysophosphatidylcholine, phosphorylcholine, tripolyphosphate, phosphorylserine, phosphoglyceric acid, inositol monophosphate, inositol biphosphate, inositol triphosphate, inositol tetraphosphate, inositol pentaphosphate, inositol hexaphosphate, and phosphorylinositols; i.e., phosphate esters and phosphate anhydrides. In addition, lipoteichoic acids, teichoic acids (including pneumococcal and streptococcal C-polysaccharide), sulfatides, nucleotides, stearyl amines, diacylglycerol, aminocaproic acid, pyridoxal phosphate, chondroitin sulfate, cysteic acid, p-aminophenyl phosphate, p-aminophenyl sulfate, poly-l-lysine, poly-l-arginine, poly-l-glutamic acid, poly-l-aspartic acid and other polymeric and monomeric amino acids will efficaciously reduce toxicity.

Further, phospholipids such as cardiolipin, phosphatidylinositol phosphates, including phosphatidylinositol phosphate and phosphatidylinositol diphosphate, phosphatidylcholine, phosphatidylserine, phosphatidylglycerol, galactocerebroside sulfate, phosphatidylinositol and phosphatidic acid, all of which will spontaneously form liposomes at excess concentrations in water (above their critical micelle concentrations) would also be expected to reduce aminoglycoside toxicity. Such aminoglycoside-containing liposomes would allow aminoglycosides to be given safely above the LD₁₀ or



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LD₅₀ of the free drug. For example, the LD₅₀ of streptomycin in mice is 741 mg/kg (see Table IV infra); encapsulation of streptomycin in liposomes would allow the administration of this concentration of streptomycin without observing any acute toxicity.

In a particular embodiment, the phospholipids phosphatidylinositol phosphate and phosphatidylinositol biphosphate may be especially useful when used at concentrations greater than their critical micelle concentrations; that is, with the aminoglycoside antibiotic encapsulated within liposomes of these phospholipids. As discussed above, these phospholipids are the putative toxicity receptors for aminoglycosides, and for this reason, they are especially desirable for use as ligands in the preparations of the invention, either above or below their critical micelle concentrations (i.e., in liposomes or in solution).

The preparations of the invention may be used in situations requiring high initial concentrations and/or high levels of sustained antibiotic therapy. They may also be useful in renal compromised patients in which initial toxicity to the patient is a concern. The invention also enables administration of antibiotics at levels higher than currently acceptable levels for sustained minimal inhibitory serum concentrations. By virtue of the ability to administer higher loading dosages of antibiotics, higher sustained levels of antibiotics are possible. The technology of the invention may also be applied in veterinary therapy to situations including, but not limited to, gram-negative enteritis, mastitis, "shipping fever", infectious keratoconjunctivitis, vibrosis, mycobacterial infections and Brucella spp. infections.



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The aminoglycoside antibiotics which may be used in the present invention include but are not limited to: streptomycin, gentamycin, tobramycin, amikacin, kanamycin and neomycin.

5

According to the present invention, an aminoglycoside antibiotic is mixed with a ligand prior to administration to an organism in vivo. Sterile water is the preferred diluent. Due to the nature of the aminoglycoside antibiotics, parenteral administration of the preparation is the preferred route. The aminoglycoside and ligand may be mixed immediately prior to administration in vivo. Alternatively, the aminoglycoside and ligand may be mixed for 1 hour, 2 hours, or longer prior to administration, depending on the stability of the aminoglycoside. Using either method, toxicity is reduced and the antibiotic retains antimicrobial activity. As a result, increased doses of antibiotic may be administered to animals or humans with greater safety.

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The reasons for the attenuation of toxicity remain obscure, and a number of mechanisms seem plausible. Possibly the ligands form a complex with the aminoglycosides, thus preventing the binding of the antibiotic to its putative aminoglycoside toxicity receptor in vivo. It is also conceivable that association between aminoglycosides and ligands occurs by virtue of dipole interactions, resulting in an array of hydrogen bonds between them. At physiological pH, the guanidino groups of aminoglycosides are fully protonated and are stabilized by resonance. These groups are particularly good sites for hydrogen bond interactions with phosphate groups (examples of which are the binding of NAD^+ to arginine 101 of lactate dehydrogenase or the binding of

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thymidine 3'-5' diphosphate to the guanidinium ions of arginines 35 and 87 of staphylococcal nuclease). Guanidino groups are capable of forming up to five hydrogen bonds each, and can interact simultaneously with phosphate groups and adjacent groups such as carbonyls. Alternatively, the ligands may bind to the putative aminoglycoside toxicity receptors themselves, thus preventing the binding of the antibiotic.

4.1.1. EVIDENCE FOR COMPLEX FORMATION

The following observations and data suggest that an aminoglycoside-ligand complex is formed which is less toxic than the aminoglycoside alone:

(1) SDS-Polyacrylamide Gel Electrophoresis:

Equal volumes of mouse serum were incubated for 0.5 hour and 2 hours with ^{125}I -gentamycin sulfate (^{125}I -GS) or with ^{125}I -gentamycin-phosphorylcholine (^{125}I -GPC), 1:3 molar ratio, at 1×10^5 cpm per sample. Aliquots of the incubated serum samples and aliquots of ^{125}I -GS as well as ^{125}I -GPC were then prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by boiling the samples in β -mercaptoethanol (β -ME), SDS, and urea for 1 to 2 minutes as described by Laemmli (1970, Nature 227:680). After electrophoresis the gel was stained with Coomassie-Blue (C-Blue) and exposed to X-ray film (autoradiographed) at -70°C . In the lane representing the serum sample incubated with ^{125}I -GS and in the lane representing the serum sample incubated with ^{125}I -GPC all serum polypeptides were stained with C-Blue but none of the serum polypeptide bands were radioactively labeled. However, in the lane representing the serum sample incubated with ^{125}I -GPC a low molecular weight band (i.e., lower than the molecular weights of the



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serum polypeptides) stained intensely with C-Blue; this band was also radioactive as determined by autoradiography. This low molecular weight band was not present in the lane representing serum incubated with ^{125}I -GS. In the lane representing ^{125}I -GS (without sera) no bands were detected by autoradiography, but in the lane representing ^{125}I -GPC (without sera) the same low molecular weight, radioactively-labeled band which stained with C-Blue was detected.

Since phosphorylcholine stains intensely with C-Blue, this low molecular weight band which is stained by C-Blue and is also radioactively labeled represents the putative ^{125}I -gentamycin-phosphorylcholine complex. Interestingly, the putative complex did not dissociate under the denaturing conditions used in the sample preparation for SDS-PAGE.

(2) Dissociation of the Putative Complex: If the aminoglycoside-ligand mixture is prepared or administered in an appropriate "high-salt" solution (a high salt concentration has been shown to inhibit formation of complexes or cause dissociation of some complexes), the toxicity of the aminoglycoside is not reduced. One explanation for this observation is that dispersing the aminoglycoside-ligand mixture in 0.6M ammonium acetate dissociates the complex formed between the aminoglycoside and the ligand. In support of this, see Schacht, 1978, J. Lipid Res. 19:1603-1607, where 0.6M ammonium acetate is reported to elute phosphatidylinositol phosphate which was bound to neomycin immobilized on a column. When an aminoglycoside-ligand preparation dispersed in 0.6M ammonium acetate is administered subcutaneously to mice, it appears that toxicity is not significantly reduced (see Table I).



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(3) Separate Administration of Ligand and

Aminoglycoside: If the ligand is administered to an animal before the the aminoglycoside is administered, the toxicity of the aminoglycoside is not reduced to the same extent that a mixture of streptomycin and phosphorylcholine reduces toxicity (see Table I).

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Table ICOMPARISON OF TOXICITY OF STREPTOMYCIN
ADMINISTERED WITH AND WITHOUT LIGAND

5	<u>Treatment¹</u>	<u>Lethality No. Dead/No. Inoculated</u>	<u>Percent Lethality</u>
	<u>Control</u> Streptomycin	7/8	87.5
10	<u>Aminoglycoside + Ligand²</u> (Streptomycin + Phosphorylcholine)	1/8, 0/6	7
	<u>Aminoglycoside + Ligand</u> <u>in Ammonium Acetate Buffer³</u>		
15	(Streptomycin + Phosphorylcholine)	3/6	50
	<u>Aminoglycoside Administered</u> <u>After Inoculation of Ligand⁴</u> (Min. post-inoculation)		
	0	5/8	62.5
	10	5/8	62.5
20	30	6/7	85.7

¹ In all trials the streptomycin dose was 1000 mg/kg body weight inoculated subcutaneously in Swiss Webster mice. In all trials where ligand was administered, the molar ratio of aminoglycoside to ligand was 1:3.

25 ² The aminoglycoside + ligand mixture was prepared by mixing streptomycin and phosphorylcholine immediately prior to administration.

³ The streptomycin + phosphorylcholine mixture was prepared in 0.6M ammonium acetate. Subcutaneous injection of ammonium acetate alone (0.6M) was not lethal (0/6 lethality).

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⁴ In this trial both the phosphorylcholine and streptomycin were inoculated subcutaneously at different sites after the time spans indicated.

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It appears that reduction in aminoglycoside toxicity is related to the formation of a complex between the aminoglycoside and ligand for the following reasons:

(1) drug toxicity is not significantly reduced when the aminoglycoside and ligand are administered in ammonium acetate (at concentrations which are known to dissociate or prevent formation of some complexes); and (2) separate administrations of the ligand followed by drug were not effective in reducing toxicity of the streptomycin.

However, the invention is not to be limited to this theory of interaction between the aminoglycoside and ligand. Other potential explanations for the observed reduction in toxicity are discussed above.

4.2. POLYENE AND POLYENE MACROLIDE ANTIBIOTIC-LIGAND PREPARATIONS

As described in Section 2.2., the polyene and polyene macrolide antibiotics are believed to exert their toxic effects in humans and animals by binding to cholesterol, the putative toxicity receptor. Thus, ligands suitable for reducing the toxicity in mammals of the polyene and polyene macrolide antibiotics, such as amphotericin B, are those which prevent binding to endogenous cholesterol. Such ligands would include sterols and water soluble derivatives, thereof, and, in particular, cholesterol and its water soluble derivatives such as polyethylene glycol-cholesterol (PEG-cholesterol), cholesterol hemisuccinate (CHS), and the like. The water soluble cholesterol derivatives are of particular utility since clear solutions are highly preferred for injection.

The lipid ligands of this particular embodiment of the present invention may be prepared at a concentration below that used for micelle formation and



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are thus free of liposomes. Since liposomes are not present, there is no difficulty with respect to stability of the preparations. Furthermore, administration of cholesterol-containing liposomes could result in the deposition of the liposomes primarily in the liver and spleen, which may be undesirable. Additionally, one need not be concerned about administering a particulate preparation which may clog or reduce circulation. Thus, effective preparations of polyene or polyene macrolide antibiotics mixed with water soluble cholesterol or a cholesterol derivative, yet free of liposomes, are highly effective in reducing toxicity of the drugs.

Amphotericin B, a polyene antibiotic, is not soluble in water and, for injections, is commercially solubilized in sodium deoxycholate which also has toxic properties (Fungizone, E.R. Squibb & Sons, Princeton, New Jersey). Consequently, because of the two toxic components of the commercial pharmaceutical agent, therapeutic doses of Fungizone or amphotericin B-deoxycholate (amphotericin B-DOC) must be kept low. As will be seen in the examples following, both a reduction in toxicity and solubilization of Amphotericin B can be obtained by solubilizing the amphotericin B in a water soluble cholesterol preparation. Reduction in toxicity of an amphotericin B administration is obtained in two ways:

(1) Introduction into the system of toxic deoxycholate is eliminated since non-toxic water soluble cholesterol is used as the solubilizer; and

(2) Water soluble cholesterol, when added to amphotericin B, blocks affinity for in situ lipid binding sites on the drug, thus blocking the toxicity pathway.



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4.2.1. EVIDENCE FOR COMPLEX FORMATION

As indicated in Section 4.1., the reasons for the attenuation of toxicity seen when polyene or polyene macrolide antibiotics are administered in combination with the ligands of the present invention remain obscure. One possibility is that the ligand forms a complex with the polyene antibiotic thus preventing binding of the drug to its putative toxicity receptor.

The following observations and data suggest that such a complex is formed between amphotericin B and the ligand PEG-cholesterol.

Spectrophotometric Analysis: The absorbance spectrum of amphotericin B in water demonstrates three peaks at the following wavelengths: 386, 407, 418 nm. When Fungizone is mixed with PEG-cholesterol in an aqueous solution, the peak at 407 nm disappears. The absence of an absorption peak at about 407 nm is evidence of the amphotericin B-cholesterol complex formation. However, the absorbance spectra of Fungizone in methanol and Fungizone mixed with PEG-cholesterol in methanol are identical. This indicates that no complex is formed in methanol (See Section 7 for details).

4.3. ADRIAMYCIN-LIGAND PREPARATIONS

The cardiotoxicity of adriamycin appears to be mediated by binding of the drug to cardiolipin or cardiolipin head groups (see Section 2.3.). Accordingly, these preparations of the present invention utilize adriamycin in admixture with ligands which block the binding of adriamycin to endogenous cardiolipin. Such



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ligands would include cardiolipin or cardiolipin head groups.

5 As with the polyene and polyene macrolide-ligand preparations of Section 4.2., the preparations of the invention do not contain liposomes. Hence, none of the disadvantages of liposome administration are encountered with the preparations of the invention.

10 4.4. CISPLATIN-LIGAND PREPARATIONS

The nephrotoxicity and ototoxicity of cisplatin are apparently mediated in a similar fashion to that of the aminoglycoside antibiotics (see Section 2.4.).
15 Consequently, any ligand which interferes with the binding of cisplatin to phosphatidylinositol bisphosphate is suitable for buffering toxicity of cisplatin.

20 5. EXAMPLES: STREPTOMYCIN PREPARATIONS

The data presented in the following examples demonstrate the reduction of toxicity of aminoglycoside antibiotics which retain antimicrobial activity when administered as an aminoglycoside-ligand preparation.

25 5.1. REDUCED TOXICITY OF STREPTOMYCIN-LIGAND PREPARATIONS

30 The following data (presented in Table II) compare the acute toxicity of streptomycin administered subcutaneously in mice to that of streptomycin administered in combination with various ligands.

35 For each trial 200 mg of streptomycin sulfate and one of the following ligands was dissolved in distilled



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water (final volume 5 ml) to obtain a 1:3 molar ratio or a 1:1 molar ratio of streptomycin to ligand: 260 mg egg lysophosphatidylcholine (1:3); 87 mg egg lysophosphatidylcholine (1:1); 129 mg phosphorylcholine (1:3); 110 mg inositol hexaphosphate (1:1); 80 mg tripolyphosphate (1:1); 71 mg phosphorylethanolamine (1:3); 70 mg choline chloride (1:3); 93 mg phosphorylserine (1:3); 196 mg inosine 5'-monophosphate (1:3); 115 mg phosphoglyceric acid (1:3); or 76.5 mg inositol monophosphate (1:1). These solutions were mixed for at least one hour. Once dissolved, a 0.6 ml aliquot of the solution was administered subcutaneously into mice (average weight of 24 gm each) in order to deliver a dose of 1000 mg streptomycin per kg body weight.

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The results in Table II clearly demonstrate that egg lysophosphatidylcholine, phosphorylcholine and inositol hexaphosphate are very effective in reducing the toxicity of streptomycin. The remaining ligands included in Table II vary in their effectiveness in reducing aminoglycoside toxicity.

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Table II

COMPARISON OF THE ACUTE TOXICITY OF
STREPTOMYCIN ADMINISTERED
WITH AND WITHOUT VARIOUS LIGANDS¹

		<u>Lethality</u>	
		<u>No. Dead/ No. Inoculated</u>	<u>Percent Lethality</u>
<u>Control</u>			
Streptomycin		6/8, 5/6, 6/8	77.7
<u>Ligand²</u>	<u>Molar Ratio Streptomycin: Ligand</u>		
Egg Lysophosphatidylcholine	(1:3)	0/8, 0/4	0
Egg Lysophosphatidylcholine	(1:1)	0/7	0
Phosphorylcholine	(1:3)	0/8, 0/8	0
Inositol hexaphosphate	(1:1)	0/8	0
Phosphoglyceric acid	(1:3)	3/8	37.5
Tripolyphosphate	(1:1)	3/8, 4/7	47.3
Phosphorylserine	(1:3)	4/8	50
Inositol monophosphate	(1:1)	5/8	62.5
Phosphorylethanolamine ³	(1:3)	4/7	66
Inosine 5'-monophosphate	(1:3)	7/8	87.5
Choline chloride ⁴	(1:3)	7/7	100

- 1 In all trials the streptomycin dose was 1000 mg/kg body weight, inoculated subcutaneously in Swiss Webster mice.
- 2 The following compounds, when mixed with the aminoglycoside, precipitated out of solution and could not be administered to mice: egg phosphatidic acid and inositol hexasulfate.
- 3 Phosphorylethanolamine administered without streptomycin was not lethal (0/7 lethality).
- 4 Choline chloride administered without streptomycin was not lethal (0/8 lethality).



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5.2 ANTIBIOTIC ACTIVITY OF STREPTOMYCIN - PHOSPHORYLCHOLINE PREPARATIONS

5 The following experiment compares the antibiotic
activity of sera from mice inoculated subcutaneously with
streptomycin to that of sera from mice inoculated with a
streptomycin-phosphorylcholine preparation. The results
demonstrate that the toxicity of streptomycin can be
reduced (See Table II) while retaining antimicrobial
10 activity (See Table III).

Adult Swiss Webster mice were inoculated
subcutaneously with 0.6 ml of either streptomycin at a
concentration of 18.3 mg/ml (400 mg/kg body weight) or a
15 phosphorylcholine-streptomycin preparation (antibiotic as
above and phosphorylcholine 11.5 mg/ml, i.e., 255 mg/kg
body weight). At 2, 4, 6, 8.5 and 19 hours
post-inoculation, three mice from each group were
anesthetized with ether and the retro-orbital blood was
20 collected individually using heparinized capillary
pipets. After the last bleeding, the blood was
centrifuged to separate serum from cells. The antibiotic
activity of the sera was determined in 96-well, U-shaped
microplates as follows.

25 Each serum sample (50 μ l) was mixed with 50 μ l
tryptosephosphate broth. These were serially diluted in
the microplate wells (50 μ l aliquots were diluted into 50
 μ l of broth serially). Then, 50 μ l of broth containing
30 10^3 colony-forming units of Staphylococcus aureus (ATCC
No. 14154) were added to each well and the microplates
were incubated overnight at 37°C. The endpoint of the
titration was indicated by that dilution of serum in which
the growth of S. aureus in micro-well culture was

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inhibited approximately 50% as compared to the control cultures which received no antibiotics.

The results (Table III) indicate that antimicrobial activity of mouse sera obtained at different time intervals post-inoculation with antibiotic is similar regardless of whether streptomycin was administered alone or in combination with phosphorylcholine. Therefore, the antibiotic retains antimicrobial activity.

Table III

ANTIBACTERIAL ACTIVITY OF SERA FROM
MICE INOCULATED SUBCUTANEOUSLY WITH
STREPTOMYCIN-PHOSPHORYLCHOLINE PREPARATIONS

	Hours Post Inoculation	Titer ¹ in Individual Sera of Mice Inoculated with:	
		<u>Streptomycin + Phosphorylcholine</u>	<u>Streptomycin</u>
	2	2, 2, 2	2, 2, 2
	4	4, 4, 4	4, 4, 4
	6	8, 8, 8	8, 8, 8-16
	8.5	2-4, 2-4, 2	2, 2, 2
	19	2, 2, 2	0, 0, 2

¹ Reciprocal of the end-point dilution in the micro-assay described in text.

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6. EXAMPLE: REDUCED TOXICITY OF VARIOUS
AMINOGLYCOSIDE ANTIBIOTIC-
PHOSPHORYLCHOLINE PREPARATIONS

5 The following data compares the LD₅₀ of
various aminoglycoside antibiotics administered with and
without ligand.

10 In order to determine the LD₅₀ of the
aminoglycoside preparations a quantity of antibiotic was
dissolved in 20 ml distilled water with or without
phosphorylcholine to achieve a final 1:3 molar ratio of
antibiotic to ligand. Then, 0.6 ml aliquots of serial
dilutions of the aminoglycoside preparations were injected
subcutaneously into Swiss Webster mice (ten animals per
15 group, average weight 23 g each). After 24 hours the
number of survivors in each group was determined and the
LD₅₀ and 95% confidence limits for each were computed
according to the Spearman-Kärber Method for Quantal Data
(from Finney, 1952, Statistical Method in Biological
20 Assay, Hafner Pub. Co., New York). Results are shown in
Table IV.

The results in Table IV clearly demonstrate that
the LD₅₀ of each antibiotic administered in conjunction
25 with ligand is greater than the LD₅₀ of the same
antibiotic administered alone.

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Table IV

LD₅₀ OF AMINOGLYCOSIDE ANTIBIOTICS
ADMINISTERED WITH AND WITHOUT PHOSPHORYLCHOLINE

	<u>Aminoglycoside¹ Preparation</u>	<u>LD₅₀ (mg/kg)</u>	95%	<u>LD₅₀ Ratio</u>
			<u>Confidence Interval³</u> (mg/kg)	
5				
10	Streptomycin	741	616-891	---
	Streptomycin + Phosphorylcholine (1:3)	2123	1753-2570	1:2.9
15	Gentamycin	692	602-794	---
	Gentamycin + Phosphorylcholine (1:3)	1202	959-1506	1:1.7
20	Neomycin	371	301-457	---
	Neomycin + Phosphorylcholine (1:3)	912	758-1096	1:2.5

25 1 See text for explanation of subcutaneous
administration of aminoglycoside preparations in
Swiss Webster mice.

2 The LD₅₀ is expressed as mg aminoglycoside
antibiotic per kg body weight.

3 Ten animals were inoculated in each group.

30 4 The LD₅₀ ratio is the ratio of the LD₅₀ of the
aminoglycoside antibiotic to the LD₅₀ of the
aminoglycoside-ligand preparation.

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7. EXAMPLES: AMPHOTERICIN B PREPARATIONS

The following subsections describe the preparation and the formation of a complex (as evidenced by spectrophotometric data) between amphotericin B (AmB) a polyene macrolide antibiotic and PEG-cholesterol. The complex appears to remain stable in vivo. Furthermore, the complex has a reduced toxic effect on cells in vitro and when used in vivo.

7.1. INTERACTION OF AMPHOTERICIN B WITH PEG-CHOLESTEROL IN VARIOUS SOLVENTS

Because amphotericin B is insoluble in aqueous solutions, dimethylsulfoxide (DMSO) is used to solubilize the amphotericin B. The addition of PEG-cholesterol to the solubilized amphotericin B results in the formation of a complex between the amphotericin B and the PEG-cholesterol. However, DMSO, which is not approved for internal use in humans, cannot be removed by rotoevaporation. Therefore an alternate means of removal must be used. When methanol is added to the preparation, the methanol forms an easily removable azeotrope with DMSO. When PEG-cholesterol is added concurrently with the methanol, maximum complex formation is achieved.

Preparation 1: 25 mg of amphotericin B (AmB) was dissolved in 2.0 ml of DMSO. Then 30 mg PEG-cholesterol and 23 ml methanol were added to the dissolved amphotericin B. With time a precipitate formed which was removed by centrifugation at 10,000 x g for 10 minutes. The resulting supernatant was rotoevaporated to dryness at 55°C-60°C. The remaining film was suspended in 10 ml distilled water and sonicated to clearness. This preparation was designated AmB(25)/PEG-Chol.



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Preparation 2: Preparation 2 was prepared identically as for Preparation 1 except that 150 mg PEG-cholesterol was used. The preparation was designated AmB(150)/PEG-Chol-2.

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Absorbance spectra at a wavelength of 350-550 nm were obtained for the following solutions: (1) Fungizone (a commercially available preparation consisting of a lyophilized powder of amphotericin B and sodium deoxycholate) in either methanol or water ; and (2) AmB(25)/PEG-Chol and AmB(150)/PEG-Chol in either water or methanol. The spectrum obtained for Fungizone in water consisted of peaks at about 386, 407 and 418 nm. The spectra obtained for both AmB/PEG-Chol preparations in water consisted of peaks at about 387 and 417 nm; the absence of an absorption peak at about 407 nm in the spectrum is evidence of an interaction or complex formation between Amphotericin B and PEG-cholesterol. This is further supported by the fact that the spectra obtained for Fungizone in methanol and AmB/PEG-Chol in methanol are identical. The identical spectra indicates that, in methanol, amphotericin B does not form a complex with PEG-cholesterol.

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7.2. STABILITY OF AMPHOTERICIN B/PEG CHOLESTEROL

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A sample of 5 mg of AmB was dissolved in 0.5 ml of DMSO. To this was added 10 mg of PEG-cholesterol and 3.0 ml methanol. This solution was centrifuged at 12,100 x g for 10 minutes to remove a small amount of flocculate, rotoevaporated to dryness, resuspended in 10 ml distilled water adjusted to approximately pH 7.0, and sonicated to clearness. The resulting clear solution containing the AmB/PEG-cholesterol was divided into two equal aliquots (A and B).



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The following experiment indicates that the removal of PEG-cholesterol results in a loss of stability of the complex: CHCl_3 was added dropwise to aliquot A. The CHCl_3 acted to dissolve or extract the PEG-cholesterol resulting in a precipitation of amphotericin B in the aqueous phase. DMSO, when added dropwise to the precipitate, dissolved the amphotericin B in the aqueous phase.

In order to use the AmB/PEG-cholesterol complexes in vivo, it is important that the complex remain soluble in aqueous environments. To ensure that no precipitation of amphotericin B would occur in an aqueous environment, aliquot B was added dropwise to a 5% dextrose solution which is similar to plasma in osmolality. No precipitate resulted.

For purpose of comparison, amphotericin B dissolved in DMSO was added to a 5% dextrose solution. An immediate precipitate formed. Therefore, although DMSO solubilizes amphotericin B, such a solution cannot be used in vivo because the DMSO-solubilized amphotericin B will precipitate out in plasma.

7.3. REDUCED TOXICITY OF AMPHOTERICIN B LIGAND PREPARATIONS

The toxicity of the amphotericin B preparations were evaluated in mice.

7.3.1. TOXICITY OF PEG-CHOLESTEROL

Six male Swiss-Webster mice weighing 27 gms each were given a single intravenous injection of 400 mg/kg



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PEG-cholesterol. No overt signs of toxicity were demonstrated by the mice over a one-month period of visual observation.

5 7.3.2. COMPARISON OF TOXICITY OF AMPHOTERICIN B
AND AMPHOTERICIN B/PEG-CHOLESTEROL

10 Solutions of Fungizone and solutions of
AmB/PEG-cholesterol were prepared such that the
concentrations of amphotericin B ranged from 0.625 mg/ml
to 1.25 mg/ml. These preparations were inoculated
intravenously (in approximately 0.2 ml injections) in male
Swiss Webster mice (average weight approximately 35 g).
The animals were observed for survival. The percent
15 mortality was plotted against the log dose to determine
the LD₅₀ for Fungizone and for the AmB/PEG-cholesterol
preparations. The LD₅₀ of Fungizone was 3.8 mg/kg,
whereas the LD₅₀ of AmB/PEG-cholesterol was 10.0 mg/kg.
Therefore, the AmB/PEG-cholesterol preparation is less
20 toxic than the Fungizone. In fact when compared to
Fungizone, the effective dose of the amphotericin B can be
increased about 2.5 times when the amphotericin B is
administered with the PEG-cholesterol.

25 7.3.3. TOXICITY OF VARIOUS AMPHOTERICIN B
PREPARATIONS IN MICE

30 To determine the toxicities of various
amphotericin B preparations in mice, the following
solutions were prepared in distilled water and
administered intravenously in mice:

- (1) 7 mg/ml of AmB plus 50 mg/ml of
PEG-cholesterol (PEG-Chol);
(2) 7 mg/ml AmB plus 7 mg/ml deoxycholate (DOC);
(3) 7 mg/ml AmB plus 7 mg/ml DOC plus 9 mg/ml
35 PEG-Chol; and
(4) 7 mg/ml DOC.



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Each solution was inoculated into the tail vein of female adult Swiss Webster mice (in groups of four mice, weight 23 g each). The animals were then observed for appearance of blue tail which may indicate a breakdown of the complex, resulting in the precipitation of amphotericin B and subsequent blockage of the blood vessel.

The results of the treatment are shown in Table V.

TABLE V

CONDITIONS OF MICE FOLLOWING ADMINISTRATION
OF VARIOUS AMPHOTERICIN B PREPARATIONS

<u>Group</u>	<u>Inoculum</u>	<u>Appearance of Blue Tail in Each Group of 4 Mice¹</u>
1	AmB + PEG-Chol	----
2	AmB + DOC	++++
3	AmB + DOC + PEG-Chol	--+
4	DOC	---*

1 "+" designates one mouse with discoloration extending the length of the tail.

"-" designates one mouse with no tail discoloration.

"*" designates one mouse with discoloration limited to the tip of the tail.

The appearance of blue tail in the mice is evidence of precipitated material clogging an artery in the tail. Thus, in Group 1, the total absence of blue



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color in tails indicates that a solution of amphotericin B and PEG-cholesterol forms a stable complex; i.e., one which does not precipitate out of serum. For group 2, blue tails in all the mice indicate that a solution of amphotericin B and deoxycholate precipitates out of sera and blocks the tail artery. A lower incidence of blue tails in Group 3 indicates that PEG-cholesterol lessens the amount of precipitation of an AmB-DOC solution. Finally, deoxycholate by itself (as shown for Group 4) is responsible for a limited blue tail effect.

Thus, the results in Table V tend to show that the mechanism of toxicity could be mediated through binding in situ to low affinity lipid toxicity receptors in the tail and that this toxicity can be attenuated (as shown for Group 1) by preincubation in vitro of the drug with its toxicity receptor.

7.3.4. REDUCED TOXICITY OF AMPHOTERICIN B PREPARATIONS IN CELL CULTURE

Five preparations of amphotericin B were studied to determine the effect each preparation had on cell growth. The preparations were as follows:

- 1) AmB/PEG-Chol (1:3.9), (AmB:PEG-cholesterol in a 1:3.9 ratio);
- 2) AmB/PEG-Chol (1:14), (AmB:PEG-cholesterol in a 1:14 ratio);
- 3) AmB plus DOC (Fungizone);
- 4) PEG-cholesterol;
- 5) DOC.

The minimum inhibitory concentration (M.I.C.) of the amphotericin B preparations for C. albicans was



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determined as follows: each of the 5 preparations was serially diluted in a 96 round-bottomed well plate, using 1/2 step dilutions of drug in 50 μ l Mico-broth per well and 3 wells for each dilution step. After dilutions were completed, each well received 50 μ l broth containing 10^3 cells of C. albicans. Plates were incubated overnight at 35°C in a humidified atmosphere. The endpoint of M.I.C. was determined as the maximum dilution of the drug showing 50% growth inhibition in comparison with control well cultures containing no drug, and, therefore, demonstrating 100% growth.

Cytotoxicity for L-cells was also determined in 96 flat-well plates. The drug was diluted serially (1/2 step dilutions) in 50 μ l of Eagle's Minimum Essential Medium (MEM) containing 10% fetal bovine serum. Then each well received a 50 μ l aliquot from a suspension of 3×10^5 L-cells/ml medium. After incubation for 72 hours at 37°C in a humidified atmosphere of 5% CO₂ in air, the L-cell monolayers were fixed with 5% formaldehyde and stained with 0.02% crystal violet in 5% formaldehyde. The endpoint was determined as the maximum dilution of the drug showing 50% growth inhibition in comparison with the control well cultures (no drug, 100% growth). The results of these experiments are shown in Table VI.



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TABLE VI

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INHIBITION OF CELL GROWTH BY DIFFERENT
PREPARATIONS OF AMPHOTERICIN B

		Minimum Inhibitory Concentration of Amphotericin B (@g/ml)	
10	<u>Preparation</u>	<u>C. albicans</u>	<u>L cells</u>
	(1) AmB:PEG-Chol (1:3.9)	0.2	166.6
	(2) AmB:PEG-Chol (1:14)	0.2	166.6
	(3) AmB plus DOC (Fungizone)	0.4	62.5
	(4) PEG-Cholesterol	25,000+	500.0+
15	(5) DOC	5,000+	250.0

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The results in Table VI demonstrate that the MIC for the AmB/PEG-cholesterol preparations is approximately 2.7 times greater than the MIC for Fungizone, i.e., Fungizone inhibits cell growth at lower concentrations than AmB/PEG-cholesterol. Therefore, the AmB/PEG-cholesterol preparations are less cytotoxic than Fungizone.

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Plastic petri dishes (35 mm diameter) were seeded with 50 L-cells per dish in 2 ml MEM plus 10% fetal calf serum containing one of the amphotericin B preparations (2 dishes per amphotericin B preparation). The dishes were incubated for 10 days and the cloned cultures were stained with crystal violet as described above. The results are shown in Table VII.

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TABLE VII

EFFECT OF DIFFERENT AmB PREPARATIONS
ON CLONING EFFICIENCY OF L CELLS

	<u>Prereparation¹</u>	<u>Number of Clones</u>		<u>Size of Colonies</u>	
		<u>Number of Colonies</u>	<u>% of Control</u>	<u>Mean Diameter</u>	<u>% of Control</u>
	AmB/PEG -Chol (1:3.9)	75	78.5	1.8	72
	AmB/PEG -Chol (1:14)	66	68.8	1.8	72
	AmB plus DOC (Fungizone)	53	55.2	1.5	60
	DOC	68	70.8	2.3	92
	PEG -Chol	83	86.5	2.5	100
	Control	96	100.0	2.5	100.0

¹ All three preparations of AmB were added to a final concentration of 25 @g amphotericin B/ml medium.

The results in Table VII also confirm that the AmB/PEG -cholesterol preparations are less cytotoxic than Fungizone.

7.3.5. COMPARISON OF HEMOLYTIC PROPERTIES
OF AMPHOTERICIN B PREPARATIONS

The commercial Fungizone preparation causes a significant lysis of red blood cells whereas the amphotericin B/PEG -cholesterol preparations of the present



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invention do not. This property makes the amphotericin B/PEG-cholesterol preparations more suitable for use in vivo. Details of the experiments are described below.

5 Equal aliquots of a final suspension of 2% fresh erythrocytes were mixed with serial dilutions of either Fungizone (i.e., amphotericin B with deoxycholate), amphotericin B/PEG-cholesterol (prepared as previously described), PEG-cholesterol, or deoxycholate in phosphate
10 buffered saline free of Ca^{++} and Mg^{++} ; the mixtures were incubated at 37°C for 20 hours after which the cells were pelleted by centrifugation and the released hemoglobin in the supernatants was determined spectrophotometrically at an $\text{OD}_{550\text{nm}}$. Results are
15 presented in Table VIII.

 The results in Table VIII clearly demonstrate that the AmB/PEG-cholesterol preparation is less hemolytic than equal concentrations of amphotericin B as a Fungizone
20 preparation.

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TABLE VIII

HEMOLYTIC EFFECT OF DIFFERENT PREPARATIONS
OF AMPHOTERICIN B

	Concentration ¹ (@g/ml))	OD _{550nm}			
		AmB/DOC	AmB/PEG-Chol	DOC	PEG-Chol
	0	0.00	0.00	0.00	0.00
	1	0.25	0.03	0.03	0.03
	2	0.88	0.05	0.03	0.03
	2.5	0.28	0.06	0.03	0.03
15	15	1.5+	0.24	0.03	0.03
	30	1.5+	0.44	0.03	0.03
	60	1.5+	0.90	0.06	0.03
	125	1.5+	1.16	0.23	0.03
20	250	1.5+	1.38	0.47	0.03

¹ The concentration refers to the concentration of AmB when present in the preparation (i.e., in AmB/DOC, and AmB/PEG-Chol preparations). Concentration of the preparations containing no amphotericin B (i.e., DOC, and PEG-Chol) refers to the concentration of the PEG-Cholesterol or deoxycholate in equivalents.

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WHAT IS CLAIMED IS:

1. A drug preparation of reduced toxicity,
comprising: a mixture of (a) a pharmaceutically active
therapeutic drug which exhibits toxic effects in humans or
animals by binding to a toxicity receptor, and (b) a
ligand capable of preventing binding of the drug to the
toxicity receptor in vivo while allowing the drug to
retain its pharmaceutical activity.
2. The drug preparation according to claim 1
wherein the mixture is a solution.
3. The drug preparation according to claim 1,
wherein the ligand is a lipid or lipid head group.
4. The drug preparation according to claim 3,
wherein the toxicity receptor is phosphatidylinositol
bisphosphate.
5. The drug preparation according to claim 1,
wherein the pharmaceutically active therapeutic drug is
lipophilic.
6. The drug preparation according to claim 5,
wherein the ligand is a water soluble sterol moiety.
7. The drug preparation according to claim 6
wherein the water soluble sterol moiety is polyethylene
glycol-cholesterol.
8. The drug preparation according to claim 1,
wherein the ligand by binding to the drug prevents binding
of the drug to the toxicity receptor.



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9. The drug preparation according to claim 8, wherein the ligand is a lipid or lipid head group.

5 10. The drug preparation according to claim 9, wherein the toxicity receptor is phosphatidylinositol biphosphate.

10 11. The drug preparation according to claim 1, wherein the drug is a monoaminated or polyaminated drug.

12. The drug preparation according to claim 1, wherein the drug is an antimicrobial drug.

15 13. The drug preparation according to claim 1, wherein the drug is adriamycin.

14. The drug preparation according to claim 1, wherein the drug is an antiviral drug.

20 15. The drug preparation according to claim 1, wherein the drug is an antifungal drug.

25 16. The drug preparation according to claim 1, wherein the drug is an antibacterial drug.

17. The drug preparation according to claim 16, wherein the drug is an aminoglycoside antibiotic.

30 18. The drug preparation according to claim 17, wherein the ligand is a phosphate ester, phosphate anhydride or sulfatide capable of blocking binding of said aminoglycoside to its toxicity receptor.

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19. The drug preparation according to claim 18, wherein the toxicity receptor is phosphatidylinositol bisphosphate.

5 20. The drug preparation according to claim 17, wherein the ligand is a phosphate ester, phosphate anhydride or sulfatide capable of binding to C-reactive protein.

10 21. The drug preparation according to claim 17, wherein the ligand is a phosphate ester, phosphate anhydride or sulfatide capable of forming a hydrogen bonded, stabilized complex with said aminoglycoside antibiotic.

15 22. The drug preparation according to claim 17, wherein the ligand is a phosphate ester, phosphate anhydride or sulfatide capable of forming an electrostatic complex with said aminoglycoside antibiotic.

20 23. The drug preparation according to claim 17, wherein said ligand is lysophosphatidylcholine.

25 24. The drug preparation according to claim 17, wherein said ligand is phosphorylcholine.

25 25. The drug preparation according to claim 17, wherein said ligand is phosphorylserine.

30 26. The drug preparation according to claim 17, wherein said ligand is phosphorylglyceric acid.

35 27. The drug preparation according to claim 17, wherein said ligand is inositol monophosphate.



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28. The drug preparation according to claim 17,
wherein said ligand is inositol biphosphate.

5 29. The drug preparation according to claim 17,
wherein said ligand is inositol triphosphate.

30. The drug preparation according to claim 17,
wherein said ligand is inositol tetraphosphate.

10 31. The drug preparation according to claim 17,
wherein said ligand is inositol pentaphosphate.

15 32. The drug preparation according to claim 17,
wherein said ligand is inositol hexaphosphate.

33. The drug preparation according to claim 17,
wherein said ligand is a nucleotide.

20 34. The drug preparation according to claim 17,
wherein said ligand is phosphatidylinositol phosphate.

35. The drug preparation according to claim 17,
wherein said ligand is phosphatidylinositol bisphosphate.

25 36. The drug preparation according to claim 17,
wherein said ligand is tripolyphosphate.

30 37. The drug preparation according to claim 16,
wherein the drug is a polyene or polyene macrolide
antibiotic.

35 38. The drug preparation according to claim 37
wherein the ligand is a sterol or a water-soluble sterol
derivative capable of blocking binding of said polyene or
polyene macrolide antibiotic to its toxicity receptor.



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39. The drug preparation according to claim 38 wherein the water-soluble sterol derivative is capable of forming a stabilized complex with the antibiotic.

5 40. The drug preparation according to claim 38 wherein the water-soluble sterol is a water soluble cholesterol.

10 41. The drug preparation according to claim 40 wherein the water soluble cholesterol is polyethylene glycol-cholesterol.

15 42. The drug preparation according to claim 40 wherein the water-soluble cholesterol is cholesterol hemisuccinate.

43. The drug preparation according to claim 16, wherein the drug is a lincosamide.

20 44. The drug preparation according to claim 16, wherein the drug is a polymixin.

25 45. The drug preparation according to claim 1, wherein the drug is cisplatin.

30 46. The drug preparation according to claim 45, wherein the ligand is a phosphate ester, phosphate anhydride or sulfatide capable of blocking binding of cisplatin to its toxicity receptor.

35 47. The drug preparation according to claim 46, wherein the toxicity receptor is phosphatidylinositol bisphosphate.



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48. The drug preparation according to claim 45, wherein the ligand is a phosphate ester, phosphate anhydride or sulfatide capable of binding to C-reactive protein.

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49. The drug preparation according to claim 45, wherein the ligand is a phosphate ester, phosphate anhydride or sulfatide capable of forming a hydrogen bonded, stabilized complex with cisplatin.

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50. The drug preparation according to claim 45, wherein the ligand is a phosphate ester, phosphate anhydride or sulfatide capable of forming an electrostatic complex with cisplatin.

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51. The drug preparation according to claim 45, wherein said ligand is lysophosphatidylcholine.

52. The drug preparation according to claim 45, wherein said ligand is phosphorylcholine.

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53. The drug preparation according to claim 45, wherein said ligand is phosphorylserine.

54. The drug preparation according to claim 45, wherein said ligand is phosphorylglyceric acid.

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55. The drug preparation according to claim 45, wherein said ligand is inositol monophosphate.

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56. The drug preparation according to claim 45, wherein said ligand is inositol biphosphate.

57. The drug preparation according to claim 45, wherein said ligand is inositol triphosphate.

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58. The drug preparation according to claim 45, wherein said ligand is inositol tetrphosphate.

5 59. The drug preparation according to claim 45, wherein said ligand is inositol pentaphosphate.

60. The drug preparation according to claim 45, wherein said ligand is inositol hexaphosphate.

10 61. The drug preparation according to claim 45, wherein said ligand is a nucleotide.

15 62. The drug preparation according to claim 45, wherein said ligand is phosphatidylinositol phosphate.

63. The drug preparation according to claim 45, wherein said ligand is phosphatidylinositol bisphosphate.

20 64. The drug preparation according to claim 45, wherein said ligand is phosphorylinositol.

65. The drug preparation according to claim 45, wherein the drug is triphosphorylinositol.

25 66. The drug preparation according to claim 18, 19, 20, 21 or 22 wherein said aminoglycoside antibiotic is streptomycin or a pharmaceutically active salt thereof.

30 67. The drug preparation according to claim 18, 19, 20, 21 or 22 wherein said aminoglycoside antibiotic is gentamycin or a pharmaceutically active salt thereof.

35 68. The drug preparation according to claim 18, 19, 20, 21 or 22 wherein said aminoglycoside antibiotic is neomycin or a pharmaceutically active salt thereof.



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69. The drug preparation according to claim 18, 19, 20, 21 or 22 wherein said aminoglycoside antibiotic is kanamycin or a pharmaceutically active salt thereof.

5 70. The drug preparation according to claim 18, 19, 20, 21 or 22 wherein said aminoglycoside antibiotic is amikacin or a pharmaceutically active salt thereof.

10 71. The drug preparation according to claim 18, 19, 20, 21 or 22 wherein said aminoglycoside antibiotic is tobramycin or a pharmaceutically active salt thereof.

15 72. The drug preparation according to claim 37, wherein said polyene macrolide antibiotic is amphotericin B or a pharmaceutically active salt thereof.

20 73. A drug preparation of reduced toxicity, comprising: a solution of (a) a pharmaceutically active therapeutic drug which exhibits toxic effects in humans or animals by binding to a toxicity receptor, and (b) a phospholipid capable of preventing binding of the drug to the toxicity receptor in vivo while allowing the drug to retain its pharmaceutical activity, said phospholipid being at a concentration below the concentration needed to
25 form liposomes.

30 74. The drug preparation according to claim 73, wherein the ligand prevents binding of the drug to phosphatidylinositol bispophate.

35 75. The drug preparation according to claim 73, wherein the drug is an aminoglycoside antibiotic.

76. The drug preparation according to claim 75, wherein the phospholipid is cardiolipin.



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77. The drug preparation according to claim 75,
wherein the phospholipid is phosphatidylinositol phosphate.

5 78. The drug preparation according to claim 75,
wherein the phospholipid is phosphatidylinositol
bisphosphate.

10 79. The drug preparation according to claim 75,
wherein the phospholipid is phosphatidylcholine.

80. The drug preparation according to claim 75,
wherein the phospholipid is phosphatidylserine.

15 81. The drug preparation according to claim 75,
wherein the phospholipid is phosphatidylglycerol.

82. The drug preparation according to claim 75,
wherein the phospholipid is galactocerebroside sulfate.

20 83. The drug preparation according to claim 75,
wherein the phospholipid is phosphatidic acid.

25 84. The drug preparation according to claim 73,
wherein the drug is adriamycin.

85. The drug preparation according to claim 84,
wherein the ligand is cardiolipin.

30 86. The drug preparation according to claim 13,
wherein the ligand is a cardiolipin head group.

87. The drug preparation according to claim 84,
wherein the ligand is phosphatidylserine.

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88. The drug preparation according to claim 13, wherein the ligand is phosphorylserine.

5 89. The drug preparation according to claim 84, wherein the ligand is phosphatidic acid.

90. The drug preparation according to claim 13, wherein the ligand is tripolyphosphate.

10 91. The method for administering a drug preparation of reduced toxicity, comprising: administering to an animal or human a therapeutically effective amount of a solution of claim 2.

15 92. A method for safely administering a drug at concentrations greater than the LD_{10} of said drug, comprising: administering to an animal or human a dose of a drug preparation of reduced toxicity, comprising a mixture of (a) a pharmaceutically active therapeutic drug
20 which exhibits toxic effects in humans or animals by binding to a toxicity receptor, and (b) a phospholipid capable of preventing binding of the drug to the toxicity receptor in vivo, wherein said phospholipid is administered at a concentration above its critical micelle
25 concentration, and said dose is greater than the LD_{10} of said drug.

30 93. The method according to claim 92, wherein said dose is greater than the LD_{50} of said drug.

35 94. A method for safely administering a polyene or polyene macrolide antibiotic for the treatment of fungal infections, comprising: administering to an animal or human having a fungal infection a therapeutically effective amount of a drug preparation, comprising a



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mixture of (a) a polyene or polyene macrolide antibiotic and (b) a cholesterol or sterol or a water soluble derivative thereof.

5 95. The method according to claim 94, wherein
the polyene macrolide antibiotic is amphotericin B.

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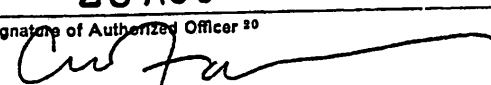
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INTERNATIONAL SEARCH REPORT

International Application No PCT/US84/00855

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC ³ A61K 9/40, A01N 25/00, A23G 3/30, C07H 15/24, C07H 15/22 A61K 31/70, A61K 35/78, A61K 31/28		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	424/10, 38, 180, 199, 287, 177 536/6.4, 6.5, 13.6, 13.7 13.2	
Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in the Fields Searched ⁵		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category [*]	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X, Y	US, A, 3,993,754, published 23 November 1976, Rahman et al.	1-13, 16-36, 66-71, 73-95
X, Y	US, A, 4,075,324, published 21 February 1978, Thizy et al.	1-12, 15, 16, 73, 74, 91-95
Y	US, A, 4,119,724, published 10 October 1978, Thizy et al.	1-12, 15, 16, 73, 74, 91-95
Y	US, A, 4,160,827, published 10 July 1979 Cho et al.	1-12, 15, 16, 73, 74, 91-95
Y	US, A, 4,256,632, published 17 March 1981 Levin et al.	1-13, 16-36, 66-71, 73, 74-95
X, Y	US, A, 4,263,428, published 21 April 1981 Apple et al.	1-13, 16-36, 66-71, 73, 74-95
Y	US, A, 4,271,196, published 2 June 1981 Schmidt	1-95
Y	US, A, 4,272,525, published 9 June 1981	1-12, 15, 16
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents: ¹⁵</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ¹		Date of Mailing of this International Search Report ²
20 August 1984		23 AUG 1984
International Searching Authority ¹		Signature of Authorized Officer ²⁰
ISA/US		

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

	Wright	37,38-42, 72-74,91-95
Y	US, A, 4,287,187, published 1 September 1981, Hoeschele et al.	1-12,16, 45-46,73, 74,91-95
Y	US, A, 4,291,023, published 22 September 1981, Hoeschele et al.	1-12,16, 45-46,73, 74,91-95
X,Y	US, A, 4,291,027, published 22 September 1981, Hoeschel et al.	1-12,16, 45-46,73, 74,91-95

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹⁰

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This International Searching Authority found multiple inventions in this international application as follows:

SEE ATTACHMENT

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☒ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹⁰ with indication, where appropriate, of the relevant passages ¹¹	Relevant to Claim No ¹²
X,Y	US, A, 4,309,420, published 5 January 1982 Ghyczy et al.	1-12,16, 73,74,91-95
X,Y	US, A, 4,332,795, published 1 June 1982 Ghyczy et al.	1-12,16, 73,74,91-95
X,Y	US, A, 4,340,599, published 20 July 1982 Lieb et al.	1-12,14,16, 73,74,91-95
X,Y	US, A, 4,346,084, published 24 August 1982 Growdon et al.	1-12,16,73, 74,91-95
X,Y	US, A, 4,358,442, published 9 November 1982 Wirtz-Peitz et al.	1-12,16,73, 74,91-95
Y	US, A, 4,372,949, published 8 February 1983, Kodama et al.	1-95
P, X,Y	US, A, 4,396,630, published 2 August 1983 Riedl et al.	1-95
P, X,Y	US, A, 4,397,846, published 9 August 1983 Weiner et al.	1-95
P, X,Y	US, A, 4,411,894, published 25 October 1983 Schrack et al.	1-12,16,73 74,91-95
P, X,Y	US, A, 4,419,348, published 6 December 1983 Rahman et al.	1-13,16-36, 66-71,73 75-95
P, Y	US, A, 4,430,330, published 7 February 1984 Growdon et al.	1-13,16,73, 74,91-95
X,Y	N, Chemical Abstracts, Vol. 97, issued 1982 (Columbus, Ohio, USA) Nesmeyanova et al. "Interaction between alkaline phosphates and acid phospholipids in E. coli cells and synthetic membranes" See page 262, Col. 1, Abstract No. 68366g, Biokhimiya (Moscow) 1982, 47(4) 671-7	1-12,16, 44,73,74, 91-95
X,Y	N, Connors et al., "Platinum Coordination Complexes in Cancer Chemotherapy, published 1974 by Spriger-Verlay (New York-Heidelberg, Berlin) See pages 46-53	